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Office of Toxic Substances  
U.S. Environmental Protection Agency  
401 M Street, S. W.  
Washington, D. C. 20460

(A)

RE: TSCA Section 8(e) Submission for 2-Amino-4-Nitrotoluene (CAS # 99-55-8)

ATTN: TSCA Section 8(e) Coordinator

This submission is made in accordance with TSCA Section 8(e) requirements and discharges any TSCA Section 8(e) responsibilities that exist for The Procter & Gamble Company (P&G) regarding the information described herein. We do not believe the data described in this submission reasonably support the conclusion that the subject material presents a substantial risk of injury to human health or the environment.

This submission provides results from a 24 Hour Exposure Clonal Syrian Golden Hamster Embryo (SHE) Cell Transformation Assay Testing the *In Vitro* Transformation Potential of 2-Amino-4-Nitrotoluene. The results show that the test substance induced morphological changes during this *in vitro* evaluation. Corroborative, data exist which shows 2-Amino-4-Nitrotoluene to be positive in an *in vivo* bioassay and an Ames test.

P&G does not currently manufacture, import, distribute, or process 2-Amino-4-Nitrotoluene nor does the Company presently intend to use this substance for these purposes in the future. We have handled and will continue to handle this material with appropriate caution in keeping with our standard practice for handling all chemical substances.

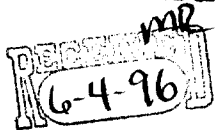
If you wish further information, please contact me at (513) 627-6145.

Very truly yours,

THE PROCTER AND GAMBLE COMPANY

W. E. Bishop, Ph.D.  
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**24 Hour Exposure Clonal Syrian Hamster Embryo (SHE) Cell Transformation Assay Testing the *In Vitro* Transformation Potential of 2-Amino-4-Nitrotoluene (CAS# 99-55-8)**

A 24 hour exposure SHE cell transformation assay was performed to test the morphological transformation potential of 2-Amino-4-Nitrotoluene. The assay was performed according to the methodology outlined in the attached protocol. In the 24 hour SHE assay, 2-Amino-4-Nitrotoluene gave significant ( $p < 0.05$ ) morphological transformation results, relative to the solvent control, at 2 of 5 tested concentrations. The trend test result for this assay was also significantly positive. According to our established criteria which states that either two positive doses or one positive dose and a positive trend test constitutes a positive SHE cell transformation assay result, 2-Amino-4-Nitrotoluene is considered positive in the 24 hour exposure SHE cell transformation assay.

A Comprehensive Protocol for Conducting the Syrian Hamster Embryo Cell Transformation Assay at pH 6.70.

(Protocol for SHE Cell Transformation at pH 6.70)

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**Keywords:** Syrian hamster embryo cells, *in vitro* cell transformation protocol, pH, carcinogen screening

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## Summary

Studies from our laboratory have demonstrated several advantages of conducting the Syrian hamster embryo (SHE) cell transformation assay at pH 6.70 compared to that done historically at higher pHs (7.10-7.35). These include reduction of the influence of SHE cell isolates and fetal bovine serum lot variability on the assay, an increase in the frequency of chemically-induced morphological transformation (MT) compared to controls, and an increased ease in scoring the MT phenotype. The purpose of this paper is to report a comprehensive protocol for conduct of the pH 6.70 SHE transformation assay including experimental procedures, a description of criteria for an acceptable assay and statistical procedures for establishing treatment-related effects. We have also identified several assay parameters in addition to pH which can affect transformation frequencies, particularly the critical role colony number per plate can have on transformation frequency. Control of this parameter, for which details are provided, can greatly increase the reproducibility and predictive value of the assay.

## INTRODUCTION

Since 1965, early passage Syrian hamster embryo (SHE) cells have been used extensively in a clonal cell transformation assay to assess the carcinogenic potential of a wide variety of chemicals and to study mechanisms of chemical carcinogenesis (discussed by Istort et al., this volume; LeBoeuf et al., this volume; LeBoeuf et al., 1989). A literature survey (Istort et al., this volume) indicates that over 470 individual chemical or physical agents and 180 chemical/physical agent combinations have been examined in the SHE assay for their ability to induce transformation. For 213 of the chemical/physical agents tested individually, rodent carcinogenicity exist. For this data set, an 80% agreement between the endpoints of transformation in SHE cells and carcinogenicity were observed, indicating the utility of this system for assessing the carcinogenic potential of chemicals. The endpoint of morphological transformation, which is most commonly used to assess a chemical's "transforming" potential, has been shown to represent an early, pre-neoplastic phenotype in this system (LeBoeuf et al., 1990; Watanabe et al., 1991). Thus, this model has several attributes which makes it a potentially useful and biologically relevant model for assessing the carcinogenic potential of chemicals.

A detailed protocol for the isolation of hamster embryo cells and the routine conduct of the SHE assay for examining chemicals for their transforming potential was described by Pienta and coworkers (Pienta et al., 1977). More recently, a survey of how this protocol is implemented in several laboratories conducting the assay was published (Dunkel, et al., 1991). With the development of an extensive experience base over the years, it has become apparent that while the assay can be conducted reproducibly by experienced labs, several aspects of the SHE assay performed at the historical pH of 7.10-7.35 have made it difficult to use for routine screening. Among these difficulties are: 1) variability in the sensitivity of different SHE cell isolates to morphological transformation, thus requiring the screening of numerous cell isolates to find a useful one (Pienta, 1977; Dipaolo, 1980; Inoue, 1980), 2) variability in fetal bovine serum lots for their ability to support SHE cell growth and transformation, thus requiring the screening of numerous serum lots (Pienta, 1977; Schuman, 1979; Tu, 1986), and 3) a

relatively low level of induction of morphological transformation by known carcinogens (Barrett and Lamb, 1985; McGregor and Ashby, 1985; Pienta, et al., 1983; Tu, 1986; Jones, et al., 1988), requiring the scoring of large numbers of colonies to achieve statistically significant treatment-related effects or the use of non-statistical methods for determining treatment-related effects. Some laboratories have, however, reported transformation frequencies in response to carcinogen treatment high enough for the application of statistical procedures (Sanner and Rivedal, 1985). Performing the assay in culture medium at pH 6.70 has overcome many of these technical difficulties (LeBoeuf and Kerckaert, 1989) while still maintaining and, in fact, improving the high predictive capacity of the assay (see LeBoeuf et al., this volume). Culture medium of pH 6.70 permits the use of virtually all SHE cell isolates and greater than 75% of fetal bovine serum lots for conducting the morphological transformation assay (LeBoeuf and Kerckaert, 1987; LeBoeuf et al., 1989). Also, culture medium pH of 6.70 increases the transformation response up to approximately 10 fold over that seen at pH 7.10-7.35 (LeBoeuf and Kerckaert, 1987; LeBoeuf et al., 1989), permitting the use of rigorous statistical procedures such as the Fisher's Exact Test for assessing a chemical's transformation potential (LeBoeuf et al., 1989).

During the course of our studies over the past several years, in addition to pH, we have identified other culture/assay parameters which, when controlled for, can further increase the reproducibility and reliability of this assay. The purpose of this report is to communicate in detail a comprehensive protocol which incorporates our learnings for conducting the SHE cell morphological transformation assay at pH 6.70.

## **MATERIALS**

All reagents are prepared in a class II biohood to maintain sterility.

### *Culture medium*

Dulbecco's Modified Eagle's Medium - LeBoeuf's modification (DMEM-L), obtained as a filter sterilized liquid from Quality Biologicals Inc. (Gaithersburg, MD), is stored at 4° C (shelf life = 6 months). DMEM-L is a modified formulation of DMEM containing low glucose (1000 mg/L), equimolar concentrations of MgCl<sub>2</sub> replacing MgSO<sub>4</sub>, a reduced concentration of phenol red (5 versus 15 mg/L), and a reduced concentration of NaHCO<sub>3</sub> (0.75 g/L or 8.9 mM versus 3.75 g/L or 44.6 mM) (LeBoeuf et al., 1989). Fetal bovine serum (FBS- Hyclone Labs Inc., Logan, Utah), is stored at -70° C until opened, and then it is stored at 4° C. L-glutamine (200mM), obtained from Gibco Labs (Gaithersburg, MD), is stored frozen at -10° C until use. To make complete culture medium (referred to as culture medium), 10 ml L-glutamine (200 mM) is added to 490 ml of DMEM-L, resulting in 4mM L-glutamine in the medium. 125 ml FBS is then added to the 500 ml DMEM-L + L-glutamine, resulting in culture medium containing 20% FBS. Bottles of complete culture medium are labeled, dated, stored at 4° C, and used within 2 weeks. This culture medium should yield a pH of approximately 6.70±, when incubated at a CO<sub>2</sub> concentration of 10% and a temperature of 37° C, in 90% humidified air. The pH of each new lot of culture medium is checked prior to its use. This involves overnight incubation of the culture medium under incubator conditions described above, followed by pH determination immediately upon removal from incubation in a septum capped vial to prevent degassing of the CO<sub>2</sub>. Occasionally the culture medium pH needs to be adjusted with NaHCO<sub>3</sub>. If adjustment is required, the amount of NaHCO<sub>3</sub> required for proper pH is determined by adding increasing volumes of 7.5% NaHCO<sub>3</sub> (Sigma) to a series of vials of culture medium, incubating them overnight at 10% CO<sub>2</sub> and measuring pH.

### *Wash solution for the cell isolation*

Wash solution is prepared by adding 10,000 units/ml penicillin-10,000 ug/ml streptomycin stock solution to 500 ml calcium-magnesium-free Hank's Balanced Salt Solution (CMF-HBSS), both obtained as sterile solutions from Gibco Labs Inc. and stored at 4° C. Bottles of wash solution are labeled, dated, stored at 4° C, and used within 30 days.

#### *Dissociation solution for the SHE cell isolation*

Trypsin (40X Enzar-T), obtained from Armour Pharmaceutical (Kankakee, IL.), is dispensed into 5 ml aliquots, which are stored frozen at  $-10^{\circ}\text{C}$  and thawed just before use. Pancreatin (4X-NFU), obtained from Gibco Labs Inc., is stored frozen at  $-10^{\circ}\text{C}$  and thawed just before use. On the day of isolation, to 282.75 ml CMF-HBSS add: 3.75 ml 40X Enzar-T trypsin (resulting in 1.25% v/v), 7.5 ml 4X-NFU pancreatin (resulting in 2.5% v/v), and 6.0 ml of 10,000 units/ml Penicillin-10,000 ug/ml Streptomycin stock resulting in 200 units/ml and 200 ug/ml, respectively. The solution pH is adjusted to neutral (red color) with 7.5%  $\text{NaCO}_3$  solution (Gibco), maintained at  $4^{\circ}\text{C}$ , and used immediately.

#### *Culture medium for the isolation*

One bottle of DMEM-L culture medium is prepared as described above and 10 ml of 10,000 units/ml Penicillin-10,000 ug/ml Streptomycin stock is added to yield 200 units/ml penicillin and 200 ug/ml streptomycin.

#### *Cryopreservation medium*

Dimethylsulfoxide (DMSO), Aldrich (Milwaukee, WI), is added to culture medium to yield 7.5% (v/v) DMSO. The solution is prepared the day of use.

#### *SHE cell detachment solution*

Trypsin (0.25% in HBSS), obtained from Gibco, is stored frozen at  $-10^{\circ}\text{C}$ .  $\text{Na}_2\text{EDTA}$  is prepared as a 2% stock solution, and stored at  $4^{\circ}\text{C}$ . To 395 ml of CMF-HBSS, 100 ml Trypsin solution (resulting in 0.05%) and 5 ml  $\text{Na}_2\text{EDTA}$  stock (resulting in 0.02%) are added. Aliquots of this solution are stored frozen at  $-20^{\circ}\text{C}$  and thawed just before use.

#### *Giemsa stain solution*

Giemsa stain stock is prepared by dissolving 1 gm powdered Giemsa (Sigma) in 66 ml glycerin (Baker) plus 66 ml methanol (Baker) and heating to  $60^{\circ}\text{C}$  for one hour. A buffer solution containing 0.3 gm



$\text{Na}_2\text{HPO}_4$  plus 0.7 gm  $\text{NaH}_2\text{PO}_4$  in 100 ml  $\text{H}_2\text{O}$  is prepared. Immediately prior to use, 20 ml of Giemsa stock is added to 100 ml buffer plus 5 ml methanol. This solution is then filtered through a 0.2  $\mu\text{m}$  filter.

#### *Other solutions and reagents*

0.4% trypan blue stain (Gibco), sterile 7.5%  $\text{NaHCO}_3$  (Gibco), isotonic buffered saline (Scientific Products, McGaw, IL), absolute methanol (Baker, Phillipsburg, NJ), and benzo(a)pyrene (Aldrich) are obtained.

## **METHODS**

#### *Hamster sacrifice/isolation of embryos*

Pregnant (13 day gestation) Syrian golden hamsters (Charles River Labs, Wilmington Colony) are killed by  $\text{CO}_2$  asphyxiation. Usually 3-4 animals are used to obtain 30-35 embryos for the cell isolation procedure. The ventral surface of each animal is rinsed with copious amounts of aqueous 70% ethanol and, using sterile instruments and technique, the abdominal skin is incised, retracted, and the peritoneal cavity is opened. Both uterine horns are removed from each animal and placed in sterile 100 mm culture dishes containing 50 ml wash solution. Dishes are placed on ice and transported to a class II biohood.

#### *Dissociation of embryonic tissue*

Using sterile instruments and technique, embryos are removed from the uteri and transferred to a second sterile culture dish with wash solution on ice and aseptically decapitated, eviscerated, and delimbed. The remaining embryo tissue is transferred to a sterile 100 mm culture dish on ice, and cut into small (approximately 1-3 mm) pieces with opposing scalpel blades or curved scissors. The tissue is transferred to a sterile trypsinization flask (Bellco, Vineland, NJ) containing a magnetic stir bar, and placed on a stir plate, where it is rinsed with 100 ml wash solution at a slow stirring speed for 5 minutes to remove as many blood cells as possible. The tissue is allowed to settle and the wash solution is

pipetted-off and discarded. This rinse step is then repeated. To begin the actual isolation, 100 ml dissociation solution is added to the flask which is gently stirred for 10 minutes. Meanwhile, 2 ml of FBS is added to a series of 50 ml sterile plastic centrifuge tubes on ice (6-8 tubes). The tissue is allowed to settle and the supernatant (dissociation solution plus cells) is carefully transferred by pipette into the prepared centrifuge tubes to a volume of approximately 50 ml. Fresh dissociation solution (100 ml) is added to the tissue and again stirred for 10 minutes. The solution plus the cells are collected as described above. The dissociation step followed by the pipette transfer of cells to serum-containing tubes is repeated 3-4 times. The resultant cell suspensions plus FBS are centrifuged at 1000 rpm for 10 minutes at 4° C and the supernatants are decanted and discarded. The cells are resuspended and pooled in 20-50 ml culture medium containing antibiotics, on ice. The viable cells are counted with a hemacytometer (by exclusion of trypan blue dye) and  $2 \times 10^7$  viable cells in 30 ml culture medium plus antibiotics are placed into a T150 culture flask. The flasks are incubated at 37° C and 10% CO<sub>2</sub> in 90% humidified air for 4 hours. The culture medium is removed and the flasks are refed with 30 ml fresh culture medium and returned to the incubator. After 24 hours, the cell growth is checked, and if the flasks are not 60-80% confluent, they are refed with fresh culture medium and allowed to grow for an additional 24 hours. Otherwise, the cells are cryopreserved as described below. If the cultures have not reached 60-80% confluence by 48 hours of incubation, they are discarded and the isolation is repeated.

#### *Cryopreservation of SHE cells*

When the cells are 60-80% confluent, the culture medium is removed from the flasks and the cells are rinsed twice (1 minute each) with 10 ml CMF-HBSS. The cells are detached with 5 ml detachment solution (detachment is observed with a phase contrast microscope). Five ml culture medium (minus antibiotics) is immediately added to the flasks, and these cell suspensions are pooled in sterile centrifuge tubes. The cells are collected by centrifugation as described above, and the supernatant is decanted and discarded, while the cells are held on ice. The cells are counted with a hemacytometer as described above and diluted with culture medium containing 7.5% DMSO to  $2.5 \times 10^6$  cells/ml. One ml

of the cell suspension is dispensed into cryostorage vials (Nunc), and the cells are frozen by placing vials into a -70° C freezer for 24 hours, prior to transfer to and storage under liquid nitrogen.

#### *Feeder cell preparation*

Cryopreserved SHE cells are reconstituted by thawing a vial of frozen cells at 37° C and transferring them into a T150 culture flask containing 30 ml of culture medium. The culture medium used in the SHE assay does not contain antibiotics. The cells in the flask are incubated at 37° C and 10% CO<sub>2</sub> for four hours, refed with 30 ml of fresh culture medium and returned to the incubator. The cells are grown for 2-4 days to achieve 50-90% confluency. The cells are then detached by rinsing twice (one minute each) with CMF-HBSS, followed by 5 ml of detachment solution. Cell detachment is viewed with a phase microscope and, when detached, the cells are suspended in 30 ml of culture medium in a T150 flask on ice. The cells are exposed to x-ray irradiation (~ 5000 rad) so that they are viable, yet are no longer capable of replication. The cells are placed into a 50 ml centrifuge tube and centrifuged in a refrigerated unit (10° C) to form a pellet. The supernatant is removed. The cell pellet is resuspended in 30 ml culture medium, and viable cells are counted using the hemacytometer method. The cell concentration is adjusted to  $2 \times 10^4$  viable cells/ml with culture medium and 2 ml of this suspension is placed into 60 mm culture dishes (15 culture dishes/concentration of test chemical plus 15 dishes each for 0.2% solvent and benzo(a)pyrene positive control groups). The cells are incubated for 24 hours. Five dishes containing only feeder cells are included in each assay to verify the inability of the feeder cells to replicate.

#### *Target cell preparation*

A frozen vial of SHE cells is reconstituted as described above for feeder cells, and seeded into a T25 flask containing 5 ml of culture medium. The cells are incubated for 24 hours, after which they are detached as described above, suspended in 5 ml of culture medium and held on ice. The cells are counted as described above, and their concentration is adjusted to 30-50 viable cells/ml in culture medium. Two ml of this cell suspension is added into each 60 mm culture dish containing x-irradiated

feeder cells. The number of target cells seeded should be adjusted to achieve an average of 35 colonies/dish in all dishes, with an acceptable range of 25-45 colonies/dish. The procedure for determining this target cell seeding density is described below. The cells are incubated for 24 hours prior to dosing with test chemical.

#### *Test chemical dosing*

Before performing the SHE cell transformation assay, test chemical solubility in an appropriate solvent must be established. The following solvents are tested in order of preference: culture medium, dimethylsulfoxide, ethanol, and acetone. Other solvents may be required if these are not suitable. An amount of test chemical is weighed into a suitable container and gradual increments of solvent are added, with gentle mixing, warming or sonication, until dissolution occurs or an evenly dispersed, workable suspension is achieved. Care must be taken to ensure photodegradation of chemical does not occur. As such, all chemical manipulations should be conducted under yellow light conditions.

On the day of dosing, test chemical stocks are prepared by dissolving or suspending the test chemical in the chosen solvent. From the stock, five serial dilutions of test chemical in solvent are prepared to achieve 500X the final culture dish concentrations. Each of these five test chemical solutions is diluted 1:250 with culture medium to yield a 2X desired final concentration, such that upon addition to the target cells, final concentrations attained are 1X in 0.2% solvent in culture medium. Each test chemical culture dish is given 4 ml of 2X test chemical in 0.4% solvent in culture medium to give 8 ml total medium/dish, 0.2% solvent. The highest concentration of the test chemical will not exceed 5 mg/ml or 10mM. Each untreated control dish receives 4 ml of culture medium. Each solvent control dish receives 4 ml of 0.4% solvent in culture medium so that the final solvent concentration in the dish is 0.2%. Each positive control dish receives 4 ml of 2X Benzo[a]pyrene (BP) concentration in 0.4% DMSO. Final concentrations of BP ranging from 2.5 - 10 ug/ml are typically used. The culture dishes are returned to incubate at 37°C and 10% CO<sub>2</sub> for either 24 hours or 7 days. For the 24-hour dosing regimen, the culture medium

containing chemical is removed and the culture dishes are refed with 8 ml fresh culture medium (minus test chemical) 24 hours post dosing, and then are returned to the incubator for an additional 7 days.

#### *Cytotoxicity assay*

Before performing the SHE cell transformation assay, a cytotoxicity assay is conducted to establish an appropriate dose range for the test chemical. The same dosing protocol selected for the transformation assay (24 hours or 7 day exposure-see below), should be used for the cytotoxicity assay. This involves exposing SHE cells to the test chemical in clonal growth for a time period (24 hours or 7 days) to a range of concentrations of test chemical. At least 6 concentrations of test chemical are used spanning several logs of concentrations (i.e., 10, 50, 100, 500 ug/ml, 1 and 5 mg/ml). A second and occasionally a third cytotoxicity is typically required to define the appropriate dose range for the transformation assay (see discussion of valid assay guidelines below).

#### *Assessment of cytotoxicity*

The effect of chemical on relative colony plating efficiency is the primary indicator used for selecting the doses for the transformation assay. However, cell number/colony data are also used to corroborate chemical effects on colony size (see below). Cell counts are done to determine the number of cells/colony for all test groups in the final cytotoxicity assay. To determine colony number/plate, after 7 days of incubation, the culture medium is removed from 10 culture dishes per test group. Two ml DPBS buffer is added to the plates and left at room temperature for approximately 20 minutes. The buffer is removed and the cells are fixed with 2 ml 100% methanol for 20 minutes, and stained for 20 minutes with 2 ml Giemsa stain solution. To determine cell number/colony in the remaining 5 dishes/test group, the culture medium is removed and the cultures are incubated twice (1 minute/each incubation) with 2 ml CMF-HBSS, detached with 0.5 ml detachment solution, and suspended in culture medium for counting with either an automated counter or a hemacytometer. Each fixed and stained culture dish is examined with a stereo microscope to count the number of colonies/dish (a colony must contain at least 50 cells). Culture dishes with only feeder cells should contain no colonies. The mean number of colonies/dish for

each test group is determined, and the mean plating efficiency for each test group is calculated (plating efficiency = mean number of colonies/dish ÷ number of target cells seeded X 100). The mean number of cells/colony for each test group is also calculated (mean number of cells/colony = mean number of cells/dish ÷ mean number of colonies/dish) based on cell counts from trypsinized cultures.

For selection of doses for the transformation studies, the top dose of test chemical should result in 50% or greater reduction in plating efficiency (even in the presence of precipitate), compared to concurrent controls, unless the number of cells/colony is decreased due to toxicity to a point which makes evaluation of colony morphology ambiguous and precludes scoring that dose level for transformation. Decreased cell number/colony, observed visually, should be corroborated by the cell number/colony measurements obtained in the cytotoxicity screen. The highest dose should not exceed 5 mg/ml or 10mM. The low dose of the assay will be the highest concentration of test chemical which results in 90-100% plating efficiency. At least three doses in between the top and low dose are included in each assay resulting in a total of 5 analyzable doses. If the test chemical causes no cytotoxicity (defined as resulting in a 10% or less reduction in RPE), 5 concentrations will be tested up to a maximum of 5 mg/ml, or maximum solubility including one precipitating concentration. Due to normal variability in toxicity encountered from one assay to another, an extra treatment group of one concentration higher than the concentration anticipated to give approximately 50% relative toxicity should be considered to ensure that appropriate levels of toxicity are achieved. This group would only be scored if the dose group projected to cause 50% toxicity did not reach this level (see below under data collection).

#### ***Adjusted target cell seeding***

An average colony number/dish of 35 with an acceptable range of 25-45 has been chosen for conduct of the assay (see Results and Discussion). This number of colonies on a 60 mm plate 1) results in good reproducibility of the spontaneous MT frequency, 2) facilitates evaluation of the MT phenotype with minimal colony overlap, and 3) creates culture conditions for optimal expression of the MT phenotype such that positive chemicals yield sufficient numbers of transformed colonies for the application of

rigorous statistical procedures for testing treatment related effects, while still maximizing the total number of colonies generated with approximately 20 culture dishes/treatment group (20 dishes/group for the transformation assay plus the 5 dishes/group for cell number analysis is the practical limit of experiment size with one experimenter conducting the study if 6-8 treatment groups are included in the study).

We have also determined that MT frequency increases with decreasing colony number/dish in the range of 25-45 colonies/dish (LeBoeuf and Kerckaert, 1987) (see Results and Discussion). While the mechanistic basis of this colony density phenomenon is not well understood, it is likely related to the relative amounts of fibronectin or extracellular matrix in the cultures, which will be a function of the total cell number on the plates. Fibronectin can affect colony morphology and suppress expression of the transformed phenotype (Yamada and Kennedy, 1979) and has been shown to decrease expression of the MT phenotype in SHE cells (Rivedal, 1982). Thus, test chemicals resulting in a 50% or greater reduction in colony number/dish when compared to controls can increase MT frequency by virtue of a decrease in colony number/dish, and thus potentially yield a positive response due to test chemical-induced toxicity, not true transforming activity. In order to test the transformation potential of a chemical accurately, a means for keeping the number of colonies/dish must be kept relatively constant across dose groups. By increasing the number of target cells seeded/test group equivalent to the reduction in plating efficiency due to the test chemical toxicity, a relatively constant colony number/dish across all treatment groups can be maintained. The colony number adjustment procedure has minimal effect on the relative toxicity of a chemical (see results). As such, chemical effects on the cells appear to be independent of the number of cells targeted in the cell density range used for this assay. Colony number adjustment is recommended for each concentration that causes, or is expected to cause if the exact same concentration is not used in the cytotoxicity assay, a 30% or greater reduction in relative plating efficiency (see Results and Discussion). The colony number adjustment procedure is as follows. From the cytotoxicity assay results, the mean number of colonies/dish, the plating efficiency and the relative plating efficiency for each test group is calculated. The percent relative plating efficiency = (test

chemical plating efficiency ÷ solvent plating efficiency) X 100. The number of target cells needed to yield a constant colony number after toxicity-induced reductions is the number of target cells seeded in controls X (100 ÷ relative plating efficiency) for each treatment group that is adjusted. The cell number in the 2 ml of cell suspension used for target cell plating is then adjusted before plating.

### *Transformation assay*

In order to generate sufficient colony numbers/treatment group (>1000) for good assay sensitivity (see discussion on statistics), for ease of handling, we typically conduct two individual identical transformation assays (trials) for each study (20 plates for the transformation assay/treatment group, 500-800 colonies per trial). Other laboratories may find it more convenient to conduct the assay in one large experiment. No statistical analysis are done on individual trial data to test for treatment-related effects. Data from the trials are pooled for the final study analysis to determine test chemical effect on transformation frequency. Each trial includes at least 5 test chemical dose groups, a solvent control group, and at least 1 benzo(a)pyrene or other positive control group. It is also recommended that for screening purposes where maximum sensitivity is desirable, both 24 hour and 7 day test chemical exposure protocols are used. The justification for this recommendation is provided in the accompanying paper (LeBoeuf et al., this volume) and is briefly discussed below (see Results and Discussion).

For each trial, feeder cells, target cells, and dosing solutions are prepared and used as described in the cytotoxicity section above. After dosing, the culture dishes are incubated (undisturbed) for 7 days. The 24-hour exposure protocol involves removing the test chemical/solvent dosing solutions from the culture dishes 24 hours after dosing and refeeding the cultures with 8 ml/dish of culture medium minus test chemical. Control groups (nontreated, solvent, and benzo(a)pyrene) are also refed at this time. Following refeeding, cultures are incubated for 6-7 days. For some test chemicals an extra day of incubation is needed to overcome retarded colony growth as a result of refeeding. This can be determined by evaluating a culture dish on day 6 of the expression period from each treatment group



with a phase contrast microscope in order to confirm sufficient colony size for scoring. Control groups should be handled similar to chemically-treated groups.

#### *Fixing and staining of SHE cell colonies and cell counts*

Following incubation, the culture medium is removed from each dish and in 20 dishes/test group the SHE cell colonies are fixed with methanol and stained with Giemsa as described above for the cytotoxicity assay. In the remaining 5 dishes/test group, cells are detached and counted.

#### *Data collection*

Each culture dish is examined with a stereo microscope in order to count and record the number of colonies/dish. Each colony is evaluated and scored to be either normal or morphologically transformed (MT). Normal colonies contain cells in a monolayer with an organized, often flowing, pattern of growth with minimal cell stacking, particularly where the cells are at a confluent density. However, some normal colonies may exhibit a random growth pattern at the outermost perimeter of the colony, where the cells are not confluent. Morphologically transformed colonies contain cells in an extensive random-oriented, three dimensional, stacked growth pattern, with criss-crossing cells at the perimeter and in the center of the colony. Cells in MT colonies frequently are more basophilic relative to their normal counterparts (LeBoeuf and Kerckaert, 1987). Representative low, medium and high cell density transformed and normal colonies are shown in Figure 1.

#### *Guidelines for individual trials*

To ensure consistency in results from trial to trial and from experiment to experiment, several general guidelines should be followed:

1. The plating efficiencies of solvent, and positive control groups should be at least 20%. Plating efficiencies below 20% under the pH 6.70 conditions may indicate problems with the cells or media components. The average plating efficiency in our lab for controls now runs between 40 and 45%.

2. The average colony number/plate for solvent controls should be between 25-45 with an ideal target of 35. As discussed below, this results in consistent MT frequencies in controls from trial to trial and minimizes the possibility of artifacts due to changes in colony number per plate by the test chemical. For all treatment groups with a 30% or greater reduction in RPE, the colony adjustment procedure should be performed and adjusted data used in the analysis.
3. MT frequencies for the nontreated and solvent control groups typically range (90% of our values) from 0.06 - 0.7% (see Results and Discussion). If control frequencies are consistently greater than 0.7%, problems with some aspect of the culture conditions or scoring criteria may be occurring.
4. MT frequencies for the benzo[a]pyrene control groups (2.5-10 ug/ml) typically range (90% of our values) from 0.9 - 2.6% (see Results). If control frequencies are consistently below 0.9 %, problems with either some aspect of the culture conditions or the chemical may be occurring.

#### *Calculations*

For each test chemical dose and control group, the following values are calculated for each trial and for the results from combined trials for a study:

1. Mean number of colonies/culture dish.
2. Total number of colonies/test group.
3. Mean plating efficiency (PE)  $\pm$  SEM. where
 
$$PE = (\text{number colonies/dish} + \text{number of cells seeded}) \times 100$$
4. Relative plating efficiency (RPE) where
 
$$RPE = (\text{test group PE} + \text{solvent control PE}) \times 100$$
5. Number of morphologically transformed (MT) colonies.
6. MT frequency = (number of MT colonies + total number of colonies)  $\times$  100.
7. Mean number of cells/dish/test group.

8. Colony density = mean number of cells/dish ÷ mean number of colonies/dish.
9. Relative colony density = (colony density test group ÷ colony density of solvent control) X 100.

#### *Statistical analysis*

With pooled data from both trials, the total number of MT colonies per total colonies scored for each treatment group are compared pairwise to the solvent control using a one-sided Fisher's Exact Test (Armitage, 1971). A  $p < 0.05$  level of significance indicates a treatment related effect on MT frequency. An unstratified binomial exact permutation trend test (Cytel Software, 1992) for a significant positive dose response trend is also conducted. Scoring 1000 colonies per treatment group with an average control transformation frequency of 0.4% allows for a 4 to 5 fold increase in transformation to be detected as significant at the 0.05 level with a power of 80% (power is the probability that a statistical test will detect as significant a particular effect).

The validity of pooling data sets from multiple trials for determining treatment-related effects for SHE cell transformation data has been verified and documented in our previous studies (LeBoeuf and Kerckaert, 1989). A goodness-of-fit analysis on the medium controls, solvent controls and B[a]P controls data set reported in the accompanying paper (LeBoeuf et al., this volume) also confirms that the data across independent trials are consistent with the binomial distribution and hence pooling is valid (Cochran, 1954).

Logistic regression methods (Agresti, 1990) were used to evaluate the relationship between colony number per dish and MTF. The analysis was conducted for each of our control group data sets i.e. medium, solvent and B[a]P and was done on individual trial and complete study data sets from our historical data base.

*Guidelines for an acceptable study*

1. With pooled data from all trials, the top dose of test chemical should 1) cause at least a 50% or greater reduction in plating efficiency, compared to concurrent controls, unless the reduction in number of cells/colony due to toxicity at this top dose precludes scoring of the colonies for transformation, or 2) be a maximum testable dose, based on test chemical solubility or 3) be a maximum of 5 mg/ml or 10 mM test chemical in the cultures.
2. With pooled data from all trials, the total number of colonies/group for the control and treated groups should be approximately 1000 or more.
3. The average colony number/plate for controls should be between 25-45. For all treatment groups with a 30% or greater reduction in RPE, data from colony number adjustment groups should be used.
4. Control values should be consistent with the historical range of the laboratory. Our historical range of values are presented here as guidance. If our MTF is outside of the 90% range of values presented here, we evaluate the data set (including the positive control response) to ensure that the assay has adequate sensitivity to detect a chemically-induced response.
5. With pooled data from all trials, the transformation frequency of the benzo[a]pyrene positive control group must be statistically significantly greater ( $p < 0.05$ ) than the transformation frequency of the solvent control group, as determined by the Fisher's Exact Test.

*Criteria for judging the test chemical result*

A transformation response is considered treatment related if 1) at least two concentrations of chemical cause a statistically significant ( $p < 0.05$ ) increase in MTF relative to solvent treated controls, as indicated by a one-tailed Fisher's Exact Test conducted on pooled data from the two (or more) trials, or 2) the chemical causes a statistically significant ( $p < 0.05$ ) increase in MTF compared to solvent treated controls

at one concentration with a statistically significant ( $p < 0.05$ ) positive dose-response trend test (Thomas et al., 1977). Chemicals which cause a statistically significant increase in MTF at only one concentration without a positive trend may require additional studies to clarify the response with doses more closely spaced around the positive dose. A chemical is concluded to be positive in the SHE assay if a treatment-related response, as described above, is observed with either a 24 hour or a 7 day exposure to the test chemical.

## RESULTS AND DISCUSSION

The described protocol allows for the conduct of a reproducible assay for assessing the carcinogenic potential of chemicals as well as generating morphologically transformed colonies for mechanistic studies. Several of the key parameters which can affect the outcome of the assay and experimental control for these parameters have been identified and will be discussed.

### *Colony number per plate*

Colony number per plate is an important variable in the SHE assay. This observation was first reported in our earlier work (LeBoeuf and Kerckaert 1987) and is corroborated here. Because several of the other assay variables, such as cell isolates and serum lots, can potentially affect colony number per plate, we thought it useful to discuss this parameter first. In Figure 2, MTF is plotted as a function of colony number per plate for untreated controls (Fig. 2a), solvent controls (Fig. 2b) and benzo[a]pyrene treated cultures (Fig. 2c) from the P&G historical individual trial data base and a best fit regression line is shown. An analysis indicates that for all three groups, there is a statistically significant inverse relationship between colonies/dish and MTF as determined by regression analysis. By visual inspection, the increase in MTF with decreasing colony number is greater at 25 or less colonies per plate than in the 25-45 colonies per plate range. Additionally, as previously described, greater than 45 colonies per plate can result in the colonies coalescing which interferes with the scoring of the assay particularly in the controls.

To investigate the colony number per plate effect further, MTF was examined as a function of colony number per plate on study results (pooled data from two or more trials for each data point) for those experiments in which colony number per plate was in the range of 25 to 45. Data from our laboratory and Hazleton Laboratory were combined for this analysis (Figure 3). Again, the analysis indicates that MTF is statistically significantly inversely correlated with colony number per plate. However, the absolute magnitude of the change in MTF is less if the colony range is restricted to between 25 and 45 colonies than if the number of colonies/plate is allowed to go beyond these limits. Based on this analysis, we recommend plating the appropriate number of cells (see below) to obtain 35 colonies per plate or the mid-point of the desired range. Conducting all experiments in the 25-45 colonies/plate range also results in greater trial-to-trial and experiment-to-experiment consistency in results than otherwise would be obtained.

The observation of an effect of colony/plate on MTF also raises the possibility that chemically-induced toxicity resulting in a reduction of colony number/plate could in and of itself cause a significant increase in MTF and as such be incorrectly interpreted as a transforming chemical. To address this possibility, it is instructive to look at Figure 2a (untreated control results). Using the best fit line, if one starts with 25 colonies/plate and reduces this 50% to 12-13 colonies/plate, an increase in MTF from 0.4% to 0.8% occurs. A reduction from 30 to 15 colonies/plate results in an increase in MTF from 0.35% to 0.65%, a reduction from 35 to 17-18 colonies/plate results in an increase from 0.30% to 0.55%, and a reduction from 40 to 20 colonies/plate results in an increase from 0.22% to 0.45%. Thus, an approximate doubling in MTF occurs by decreasing colony number/plate by 50%. One might conclude from the size of the increases, that with scoring 1000 colonies, the level of sensitivity of the assay is not great enough to pick up this doubling as significant. However, a doubling of an MTF value via the colony number effect will lead to a greater "false positive" rate at some finite, non-zero rate, than would otherwise be observed in the absence of this effect.

In an attempt to determine how frequently increases in MTF occurred as a result of reduction in colony number/plate and not the inherent transforming properties of the chemical, we examined our data set of 56 chemicals reported in this volume (LeBoeuf et al., this volume). For concentrations of chemicals which caused 30% or greater reduction in relative plating efficiency, transformation results from the colony number unadjusted groups and adjusted groups, where colony number/plate was held constant, were compared. For this analysis, we had both unadjusted and colony number adjustment data sets on 100 dose groups for comparison (Table 1). Fourteen of the 100 responses (14%) showed statistically significant increases in MTF in the unadjusted group which did not occur in the colony adjusted group at the same concentration of chemical treatment. For this same set of 100 paired results, we also evaluated the effect of colony adjustment on relative toxicity induced by the test chemical. The mean  $RPE \pm SEM$  for the unadjusted group and colony adjusted group was  $61.9 \pm 1.9$  and  $55.3 \pm 1.8$  respectively. Thus it can be concluded that the decrease in transformation response observed with the colony adjustment is not due to a decreased "effect" of the chemical on the cells as indicated by cytotoxicity, but rather is more consistent with the colony number /plate effect which has been clearly demonstrated. Importantly, the adjustment procedure changed the overall call for three chemicals (Table 2). In the case of anilazine and phthalic anhydride, not cell adjusting resulted in one significant dose along with a positive trend. For 3 nitropropionic acid, not cell adjusting resulted in two significant doses. It is noteworthy that for the three chemicals for which colony adjustment had an effect on the overall call, all are non-carcinogens. Thus, 3 out 18 non-carcinogens (17%) in our data set would have been "falsely" concluded as having transforming activity in the absence of the colony adjustment procedure. Based on this analysis, we recommend the implementation of this procedure as a matter of practice when conducting the assay.

#### *Distribution of solvent and B[a]P positive MTF control values*

Shown in Figure 4 is the distribution of MTF values for culture medium, solvent and B[a]P controls from our historical data set where colony number per dish was in the range of 25-45. The mean, median and

90% range of values for the culture medium, solvent and B[a]P controls are shown in Table 3. These median values and ranges should be used as a reference point to those establishing this method in their laboratory until they can establish their own historical response data set. While we have not statistically quantitated the effect, it is our experience that the positive and negative controls for a given study move in parallel within our historical range i. e. when the solvent control MTF values are at the lower end of the historical distribution, so is the value for the B[a]P at the lower end of its distribution and vice versa. This suggests that whatever variables affect the transformation response beyond those which we have controlled, they do so across all groups. As such, this gives additional confidence that day-to-day variability in the assay will result in minimal false positive or negative responses. Additionally, the adequacy of the binomial distribution for these data was assessed by standard goodness-of-fit tests (Cochran, 1954). The results of this testing reflect on the appropriateness of statistical methods (typically the Fisher Exact test and/or the Cochran-Armitage test) used to analyze these data.

#### *SHE cell isolate and serum quality control*

SHE cell plating efficiency and, therefore, the number of colonies/culture dish can vary from one SHE cell isolate to another, as indicated by the range of values shown in Table 4. We have found targeting 60-80 cells to be optimal for most of our isolates, with plating efficiencies in the 40-50% range. Also, morphological transformation potential can vary from one SHE cell isolate to another, and on a rare occasion an isolate is obtained that cannot be used in the SHE assay as defined below. Therefore, the appropriate target cell seeding density and transformation potential must be determined for each new SHE cell isolate, prior to its use in the transformation assay. This is done by conducting a transformation assay of culture medium, solvent, and benzo[a]pyrene test groups (20 dishes/group) while varying the target cell seeding density (i.e., 60, 80, 100 target cells/culture dish). The target cell seeding density which results in 25-45 colonies/plate is then used for the transformation assay to establish the transformation properties of the particular isolate. For an isolate to be useful for standard screening purposes, it should meet the following criteria:



- 1) The plating efficiencies of untreated, solvent and positive control groups should be > 20%, giving an average of 25-45 colonies/culture dish.
- 2) The morphological transformation frequency (MTF) of the nontreated and solvent control groups should be within the 90% range of historical control values for other cell isolates for the lab (0.06 to 0.7% MTF for our laboratory).
- 3) The MTF of the B[a]P group should be statistically significantly greater than the MTF of the solvent control (one-sided Fisher's Exact Test,  $p < 0.05$ ). In addition, the MTF of the positive control should be within the 90% range of historical values for other cell isolates for the lab (0.9-2.6% MTF for benzo[a]pyrene for our laboratory).

As a point of reference, for the data set of approximately 80 studies reported here (LeBoeuf et al., this volume), plating efficiencies in the controls ranged from 24 to 71 % with an average of 44%. In the last four years, plating efficiencies in our controls have routinely been greater than 30%.

With the SHE cell isolation procedure described here, cryopreserved cells can be used for 6 to 9 months. Unpublished results from our laboratory suggest that cells maintained in the freezer greater than 9 months lose the ability to either be transformed or express the transformed phenotype.

As previously discussed, a high percentage of fetal bovine serum (FBS) lots will support SHE cell growth and morphological transformation at pH 6.70, compared to culture medium of higher pH. However, it is important to verify that the FBS used to supplement the culture medium in the SHE transformation assay yield target cell plating efficiencies and transformation frequencies in the control groups that are consistent with historical results for the laboratory and are consistent with those observed for a given cell isolate. Therefore, each new lot of FBS should be tested for its utility in the SHE assay, prior to its routine use in transformation studies. This is done by conducting a transformation assay similar to that for the SHE cell isolate validation assay above. Here, an established, acceptable SHE cell isolate is tested at an appropriate target cell seeding density (i.e., previously determined to result in 25-45

colonies/culture dish) with the culture medium, solvent, and benzo[a]pyrene control groups. An acceptable FBS lot should meet the above 3 criteria, stated for the cell isolate verification, for use in the SHE assay. A data summary of the results from our FBS screening tests is shown in Table 5. As can be seen from the range of MT frequencies observed with different lots of FBS, it is important to conduct this screening procedure to optimize assay conditions. Approximately 85% of the FBS lots screened in our lab have met the criteria for conducting the pH 6.70 SHE assay. We typically obtain sufficient serum from a suitable lot to be used for a 12-month period. Thus, while cell and serum verification experiments are essential to the reproducible conduct of the assay, they require but a small percentage of research time, if the assay is conducted routinely in the laboratory.

#### *Duration of exposure to the chemical*

Representative study results are shown in Fig. 5 and 6 from both 24 hour and 7 day exposure assays. With certain chemicals, the length of exposure time to SHE cells has been observed to affect the outcome of the pH 6.70 SHE assay and may have mechanistic implications (LeBoeuf et al., this volume). A mutagenic carcinogen, such as benzo[a]pyrene, gives a positive SHE assay result when tested in either the 7-day or 24-hour protocols (Figures 5A and 5B), over the same concentration range of test chemical in the two different exposure regimens. Non-carcinogens, such as anilazine, yield a negative SHE assay result when appropriately tested in either the 7-day or 24-hour protocols (Figures 5C and 5D). However, some carcinogens, methapyrilene as an example (Figures 6A and 6B), give a positive SHE assay result after 7 days of continuous chemical exposure and a negative result after a 24-hour exposure, and 7 days of "chemical-free" clonal expansion. While methapyrilene causes a statistically nonsignificant dose response increase in MT frequency with the 24-hour exposure, the concentration of test chemical is at least an order of magnitude greater than that showing a transformation response in the 7-day exposure assay, thus indicating a clear difference in the activity of this chemical in a 24-hour vs. 7-day exposure scenario. A similar response (7-day positive, 24-hour negative) has also been observed with the hepatocarcinogen di(ethylhexyl)phthalate in the pH 6.70 SHE assay and the skin tumor promoter, TPA (LeBoeuf et al., this volume). A positive result with a seven-day

exposure in combination with a negative result in a 24-hour exposure assay may suggest "promotion-like" activity for these chemicals. Another pattern of response is typified by the carcinogen benomyl (Figures 6C and 6D). Benomyl gives a positive SHE assay result with a 24 hour exposure and a negative response after 7 days' exposure with the doses evaluated for transformation. A seven-day exposure to 2.0 ug/ml benomyl resulted in a relative plating efficiency of 25% and the mitosis inhibiting effects of the benomyl were such that the colonies were too sparse to score for transformation. In contrast, with a 24-hour exposure, 2.0 ug/ml resulted in a relative plating efficiency of approximately 60% and produced clearly scorable colonies. It cannot be ruled out that benomyl would cause a positive response with a 7-day exposure, if a dose between 1.5 and 2.0 ug/ml, yielding acceptable levels of toxicity, could be run. However, due to the steepness of the cell survival curve and the narrowness of the chemical dose range, we were unsuccessful in conducting such an experiment. These results indicate that at least for some chemicals, SHE cells are able to tolerate higher test chemical concentrations for a short time period and then proliferate to express the morphologically transformed phenotype in culture medium without test chemical. A similar response has been observed with the carcinogens griseofulvin and nitrilotriacetate in the pH 6.70 SHE assay (LeBoeuf et al., this volume). These results indicate the value of conducting both the 7-day and 24-hour exposure protocols, if all patterns of transformation are to be detected.

## CONCLUSIONS

As presented in the accompanying paper (LeBoeuf et al., this volume), our results to date indicate an overall agreement of 85% between the pH. 6.70 assay, SHE and rodent bioassay results. The conduct of the SHE assay at pH 6.70 has overcome many of the technical difficulties which can be encountered when conducting the assay at higher pHs. Among these advantages are: 1) elimination of the need to screen numerous SHE cell isolates for their sensitivity to morphological transformation, 2) elimination of the need to screen numerous fetal bovine serum lots for their ability to support SHE cell growth and morphological transformation, 3) the frequency of morphological transformation has been increased approximately 10 fold, facilitating the use of rigorous statistical methods for judging a chemical's

morphological transformation potential, and 4) an increased ability to identify the morphologically transformed phenotype. Several parameters which affect the expression of the MT phenotype such as colony number per plate have also been identified and can now be controlled. The detailed procedures described here should facilitate the reproducible conduct of this assay.

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Table 1. Transformation results for chemicals for which colony number adjustment eliminated a statistically significant treatment-related increase in transformation frequency.

Chemical (Duration Of Exposure) Dose ug/ml	RPE (%) <sup>a</sup>	Number of MT <sup>b</sup> / Total Colonies	MTF (%) <sup>c</sup>	Fishers Exact p Value <sup>d</sup>
<b>Acid red 14 (24 Hour)</b>				
600	67	10/852	1.17	0.0096
600CA	59	11/1562	0.70	0.0740
<b>Anilazine (7 Day)</b>				
50	55	20/1429	1.40	0.0000
50CA	50	5/1098	0.46	0.1281
<b>Benomyl (24 Hour)</b>				
2.5	60	9/887	1.02	0.0204
2.5CA	36	8/1205	0.66	0.1105
<b>Chloral hydrate (24 Hour)</b>				
300	67	10/856	1.17	0.0087
300CA	56	7/938	0.75	0.0853
<b>Clofibrate (24 Hour)</b>				
125	50	13/849	1.53	0.0000
125CA	42	4/1655	0.24	0.1727
<b>EDTA (24 Hour)</b>				
100	48	9/614	1.47	0.0397
100CA	49	10/1225	0.82	0.2750
<b>4-Nitro-o-phenylenediamine (7 Day)</b>				
25	66	12/1107	1.08	0.0336
25CA	62	13/1708	0.76	0.1414

<b>3-Nitropropionic acid (24 Hour)</b>				
80	69	11/1166	0.94	0.0417
80CA	70	9/1714	0.53	0.3188
90	61	12/1025	1.17	0.0126
90CA	56	12/2049	0.59	0.2254
<b>Phenobarbital (24 Hour)</b>				
850	58	15/912	1.65	0.0005
850CA	61	8/1236	0.65	0.1601
1000	62	20/884	2.26	0.0003
1000CA	53	16/1826	0.88	0.1176
1250	49	14/701	2.00	0.0028
1250CA	41	13/1802	0.72	0.2645
<b>Phthalic anhydride (24 Hour)</b>				
300	68	9/810	1.11	0.0238
300CA	60	7/1348	0.52	0.2713
<b>Sodium fluoride (24 Hour)</b>				
75	44	15/682	2.20	0.0000
75CA	38	11/1958	0.56	0.1236

<sup>a</sup> RPE, relative plating efficiency

<sup>b</sup> MT, morphological transformants

<sup>c</sup> MTF, morphological transformation frequency

<sup>d</sup> Level of statistical significance of treatment-related effect compared to concurrent solvent control

CA = Colony number adjusted.

Table 2. Transformation Results On Chemicals For Which Colony Number Adjustment Changes The Overall Call

Chemical	Experiment	Dose ug/ml	RPE (%) <sup>a</sup>	Number of MT <sup>b</sup> / Total Colonies	MTF (%) <sup>c</sup>
Anilazine	7-day	Control	100 (60) <sup>d</sup>	5/2732	0.18 <sup>f</sup>
	Expt. 1	10	109	0/1555	0.00
		20	104	3/1481	0.20
		35	94	2/1346	0.15
		50	55	20/1429	1.40 <sup>e</sup>
		50CA	50	5/1098	0.48
3-Nitropropionic acid	24-hour	Control	100 (47) <sup>d</sup>	6/1676	0.36
		50	73	5/1226	0.41
		60	73	7/1221	0.57
		70	68 <sup>e</sup>	7/1136	0.62
		80	69	11/1166	0.94 <sup>e</sup>
		80CA	70	9/1714	0.53
		90	61	12/1025	1.17 <sup>e</sup>
		90CA	56	12/2049	0.59
Phthalic anhydride	24-hour	Control	100 (40) <sup>d</sup>	3/1091	0.28 <sup>f</sup>
		50	98	4/1412	0.28
		100	95	4/1283	0.31
		200	85	5/1138	0.44
		300	68	9/810	1.11 <sup>e</sup>
		300CA	60	7/1348	0.52
		400	63	6/898	0.67

		400CA	48	7/1822	0.38
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**a** RPE, relative plating efficiency

**b** MT, morphological transformants

**c** MTF, morphological transformation frequency

**d** Level of statistical significance of treatment-related effect compared to concurrent solvent control

**e** Statistically significantly different compared to concurrent solvent control or pooled controls from the two experiments studies

**f** Trend test positive only for the unadjusted results data set

**g** Colony adjustment not conducted on this group based on results from the cytotoxicity assay

CA = Cell adjusted.

**TABLE 3 Mean, median and 90% range values of our historical control data set**

<u>Group</u>	<u>No. of Experiments</u>	<u>Mean</u>	<u>Median</u>	<u>Lower 90% value</u>	<u>Upper 90% value</u>
Culture medium	72	0.290	0.290	0.063	0.610
Solvent control	81	0.322	0.307	0.074	0.665
B(a)P	83	1.538	1.435	0.910	2.592

TABLE 4

RESULTS OF TESTING SHE CELL ISOLATES<sup>a</sup> IN THE SHE ASSAY

	Plating Efficiency <sup>b</sup>	MTF <sup>c</sup>	Colonies/dish
	Mean ± SEM (Range)	Mean ± SEM (Range)	Mean ± SEM (Range)
Culture medium	40 ± 3.32 (29-61)	0.26 ± 0.07(0-0.64)	36 ± 1.70 (25-47)
Dimethylsulfoxide	47 ± 2.22 (29-60)	0.19 ± 0.03 (0-0.48)	37 ± 1.48 (27-47)
Benzo(a)pyrene	46 ± 1.80 (34-60)	1.59 ± 0.17 (0.83-3.15)	36 ± 1.62 (28-47)

<sup>a</sup> Pooled data from over 30 SHE cell isolates tested in our lab in the past 8 years.

<sup>b</sup> Plating efficiency = number of colonies/dish ÷ number of cells seeded X 100.

<sup>c</sup> Morphological transformation frequency.

TABLE 5

RESULTS OF TESTING FETAL BOVINE SERUM<sup>a</sup> IN THE SHE ASSAY

Control	Plating Efficiency <sup>b</sup>	MTF <sup>c</sup>	Colonies/dish
	Mean $\pm$ SEM (Range)	Mean $\pm$ SEM (Range)	Mean $\pm$ SEM (Range)
Culture medium	43 $\pm$ 5.37 (32-49)	0.18 $\pm$ 0.02 (0-0.45)	37 $\pm$ 2.14 (26-47)
Dimethylsulfoxide	44 $\pm$ 4.83 (25-64)	0.28 $\pm$ 0.66 (0-0.52)	35 $\pm$ 2.63 (25-43)
Benzo(a)pyrene	40 $\pm$ 4.76 (25-58)	1.96 $\pm$ 0.24 (1.40-2.89)	33 $\pm$ 2.45 (25-40)

<sup>a</sup> Pooled data from over 20 fetal bovine serum lots tested in our lab over the past 8 years.

<sup>b</sup> Plating efficiency = number of colonies/dish + number of cells seeded X 100.

<sup>c</sup> Morphological transformation frequency.

## FIGURES

Figure 1. Photomicrographs of morphologically normal and transformed SHE cell colonies under pH 6.70 culture conditions. Low density (a, b), medium density (c, d) and high density (e, f) morphologically transformed colonies: Low density (g, h), medium density (i, j) and high density (k, l) morphologically normal colonies.

Low magnification =8X; high magnification =27X

Figure 2. Effect Of Colony Number/Dish On MTF. MTF from individual trials from culture medium controls (Fig. 2a), solvent treated controls (Fig. 2b.) and B[a]P treated cultures (Fig. 2c) were plotted as a function of colony number/plate. 84, 141 and 143 individual trails were plotted for the culture medium controls, solvent treated controls and B[a]P treated cultures respectively. A statistically significant inverse relationship exists between MTF and colony number/plate for all three data sets as indicated by logistic regression analysis. p-Values for significance of the regression were medium control,  $p < 0.001$ ; solvent control,  $p < 0.050$ ; and B(a)P control,  $p < 0.001$ .

Figure 3. Effect Of Colony Number/Dish On MTF When Colony Number/Dish is Restricted to Between 25 and 45. MTF from final studies (pooled data from two or more trials/study) from culture medium controls (Fig. 3a), solvent treated controls(Fig. 3b.) and B[a]P treated cultures (Fig. 3c) were plotted as a function of colony number/plate. 72, 81 and 83 assay study results were available for the culture medium controls, solvent treated controls and B[a]P treated cultures respectively. A significant inverse relationship exists between MTF and colony number/plate for all three data sets as indicated by logistic regression analysis. p-values for significance of the regression were medium control,  $p < 0.002$ ; solvent control,  $p < 0.012$ ; and B(a)P control,  $p < 0.005$ .

Figure 4. Frequency Distributions Of Historical Control MTFs. The frequency of MTF values from our historical data base for culture medium controls (Fig.4a), solvent controls (Fig. 4b) and B[a]P controls (Fig. 4c) are shown. 72, 81 and 83 study results were available for this analysis for the culture medium

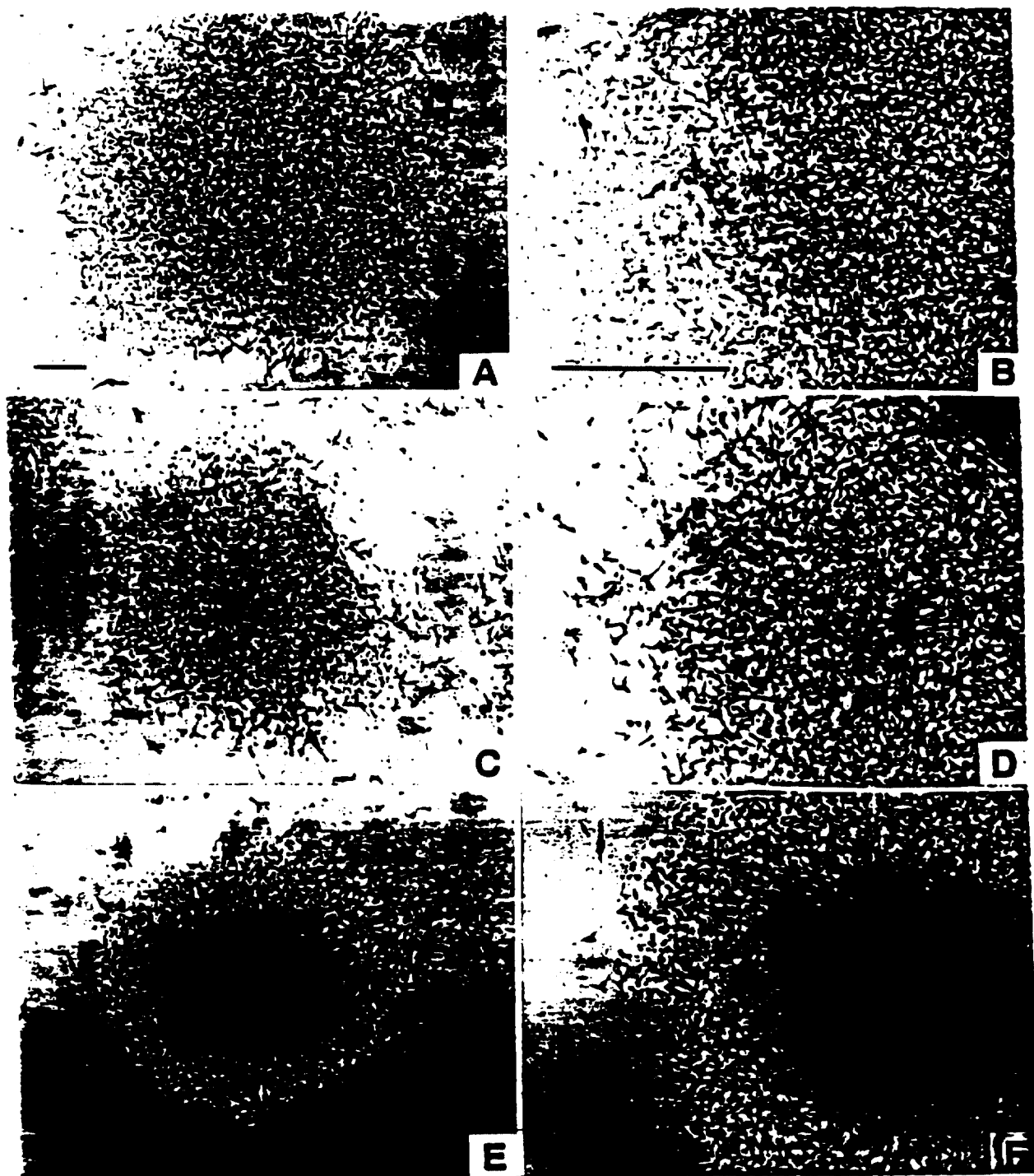


controls, solvent treated controls and B[a]P treated cultures respectively. The intervals are designed to encompass 90% of the underlying distribution of possible values (Hahn and Meeker, 1991).

Figure 5. Morphological transformation frequency (MTF) of benzo[a]pyrene (A and B) and anilazine (C and D) as a function of their toxicity (relative plating efficiency - RPE) in the 7-day and 24-hour SHE assay. Indicated dose groups (asterisks) are significantly greater ( $p \leq 0.05$ ) than the solvent control group

Figure 6. Morphological transformation frequency (MTF) of methapyrilene (A and B) and benomyl (C and D) as a function of their toxicity (relative plating efficiency - RPE) in the 7-day and 24-hour SHE assay. Indicated dose groups (asterisks) are significantly greater ( $p \leq 0.05$ ) than the solvent control group.

Fig 1



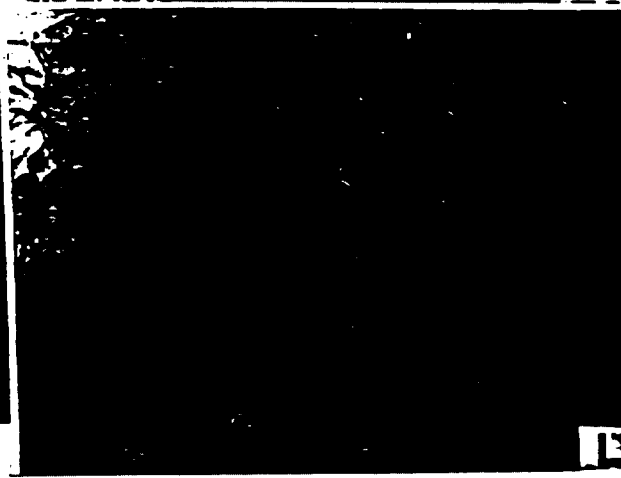
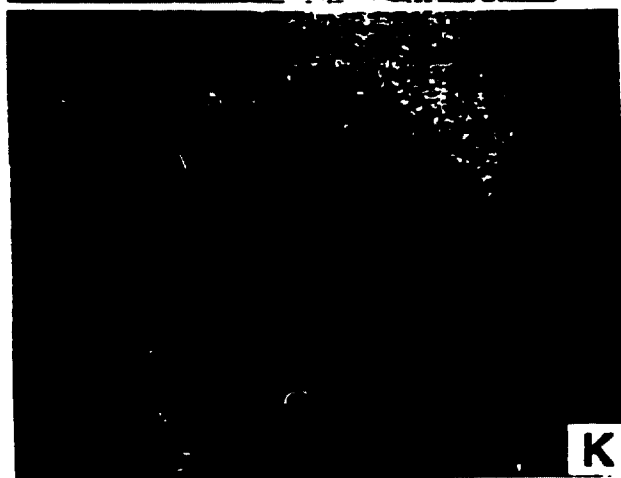
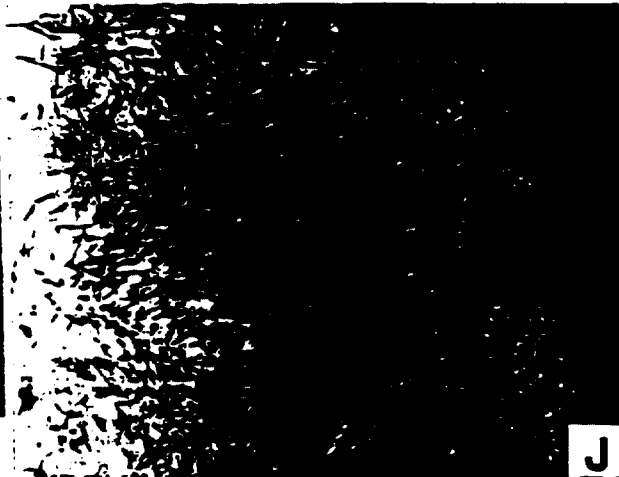


Figure 2

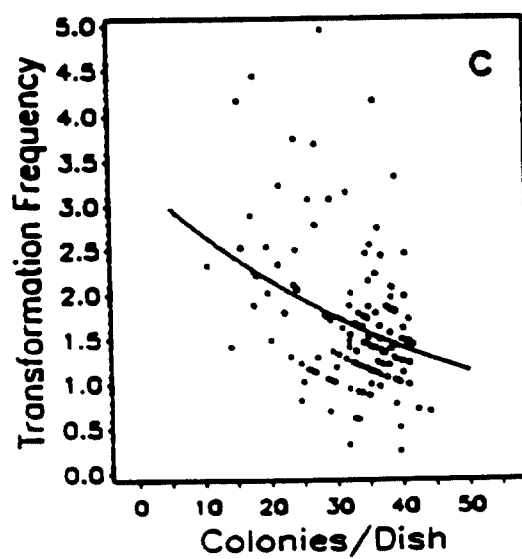
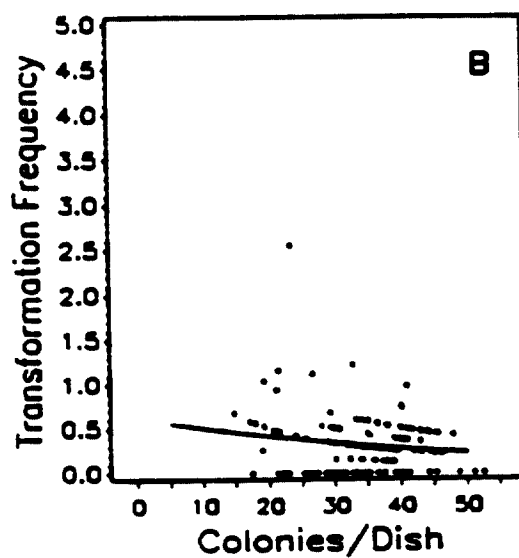
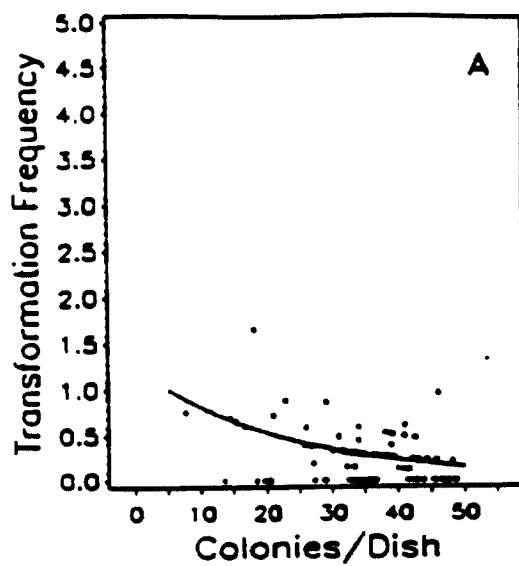


Figure 3

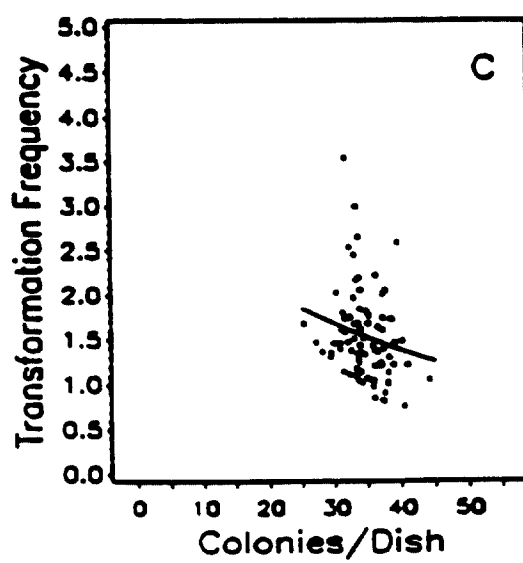
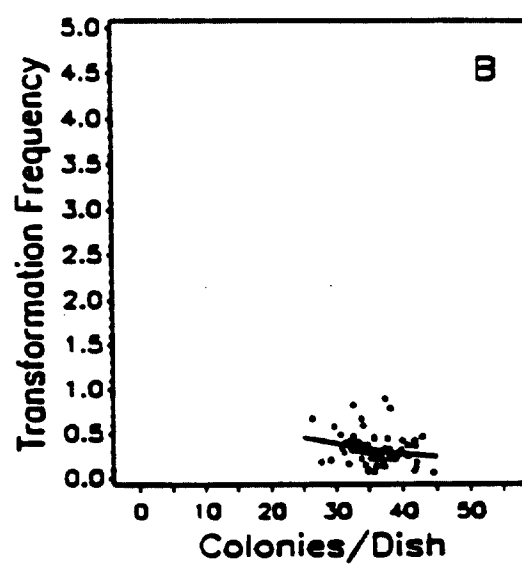
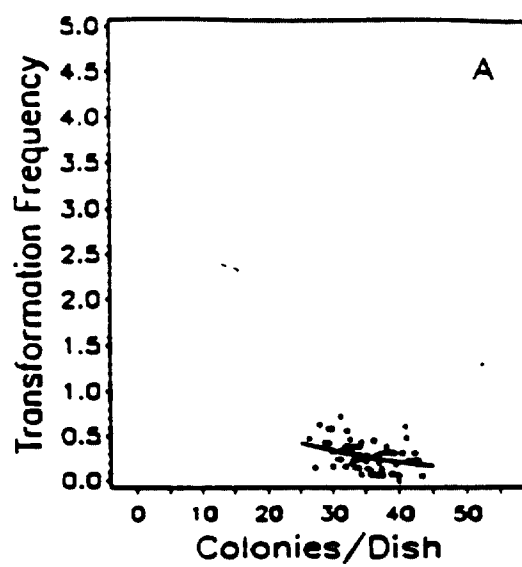


Figure 4

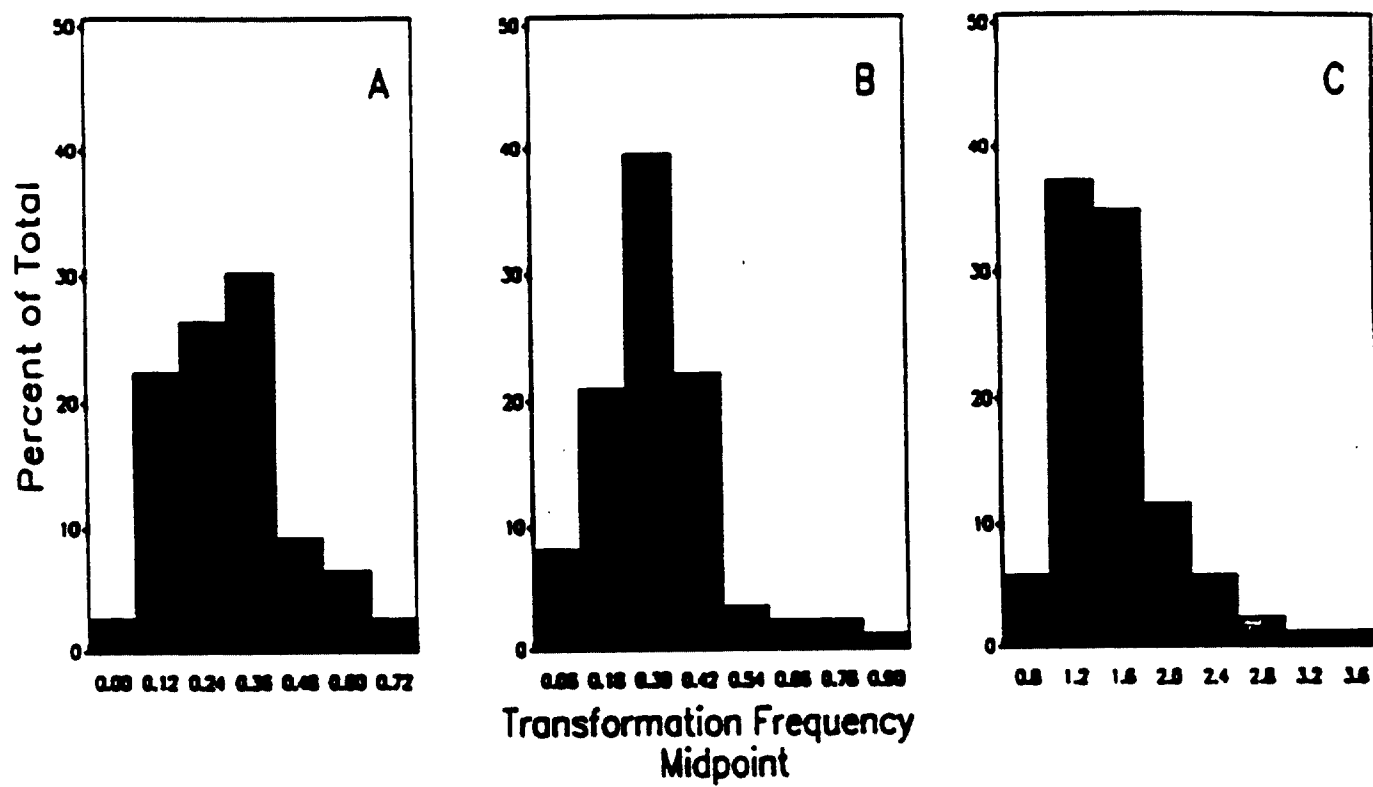


Figure 5

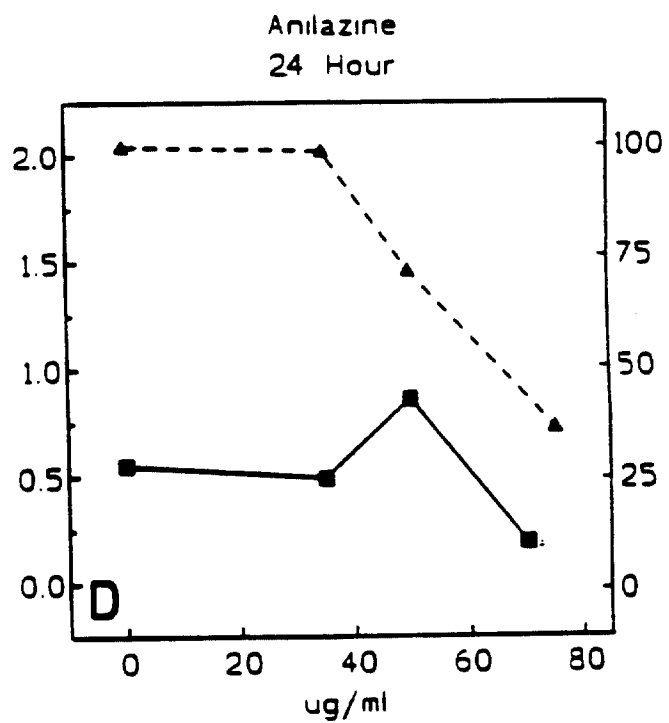
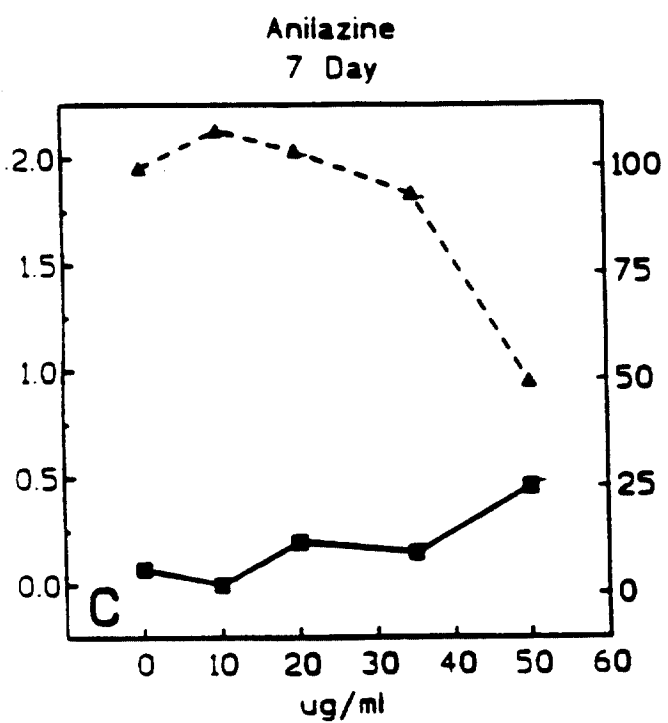
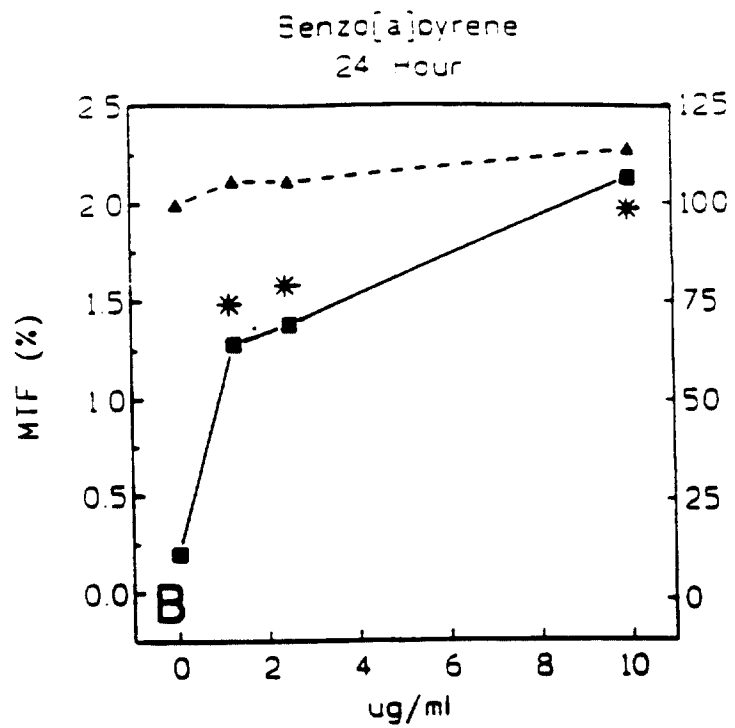
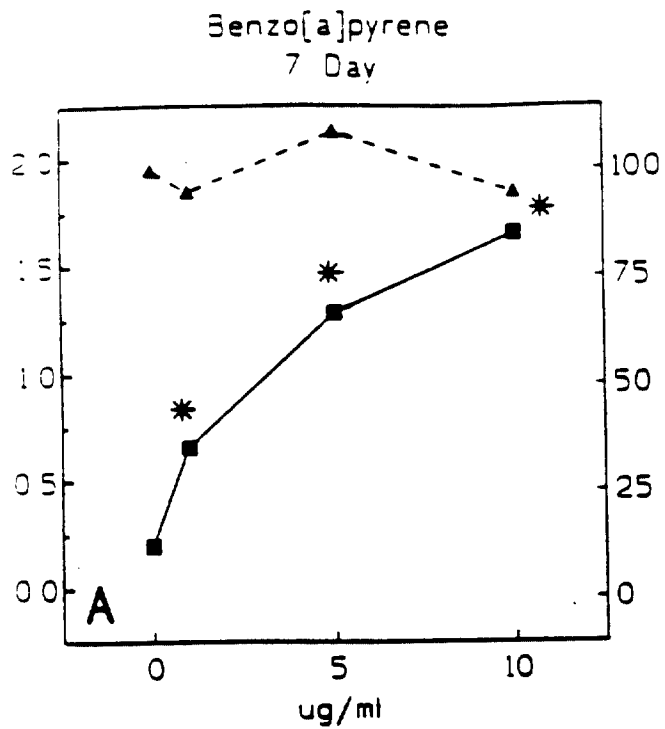
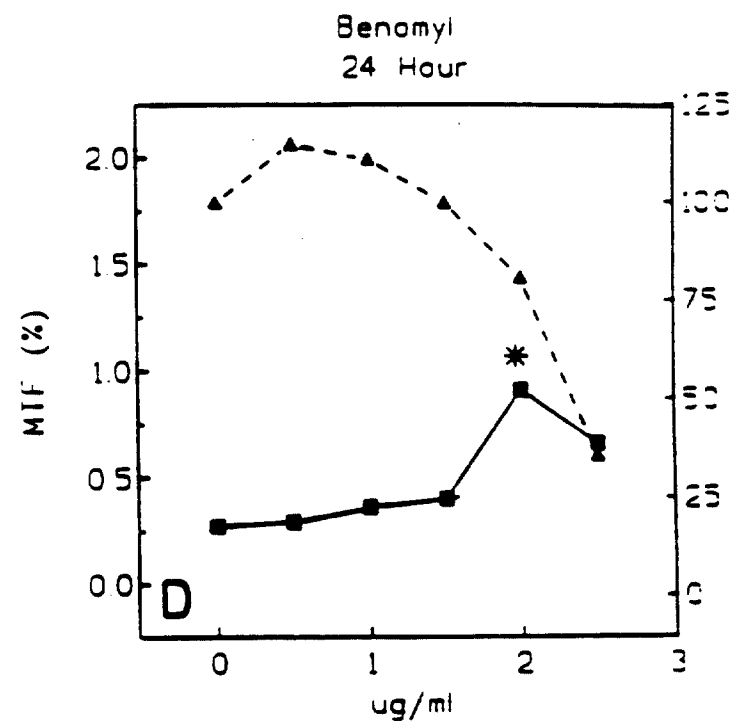
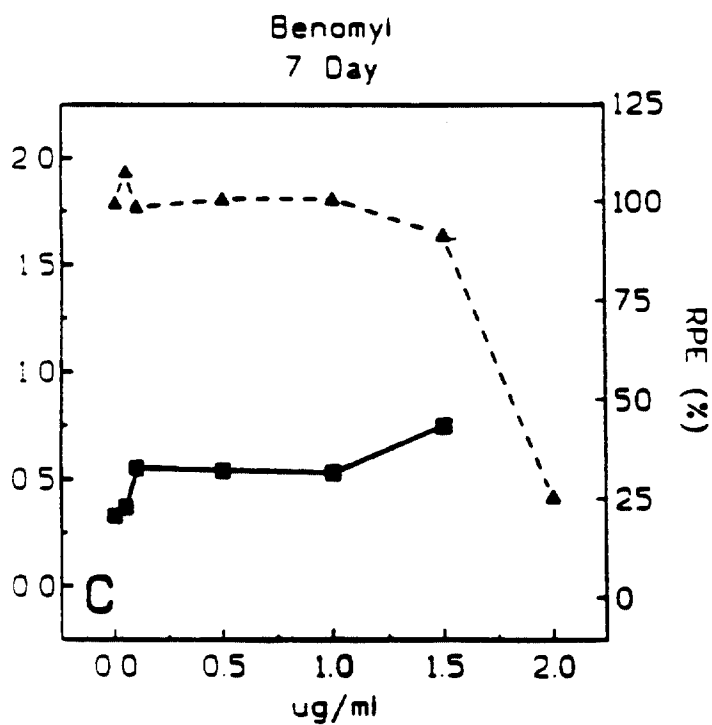
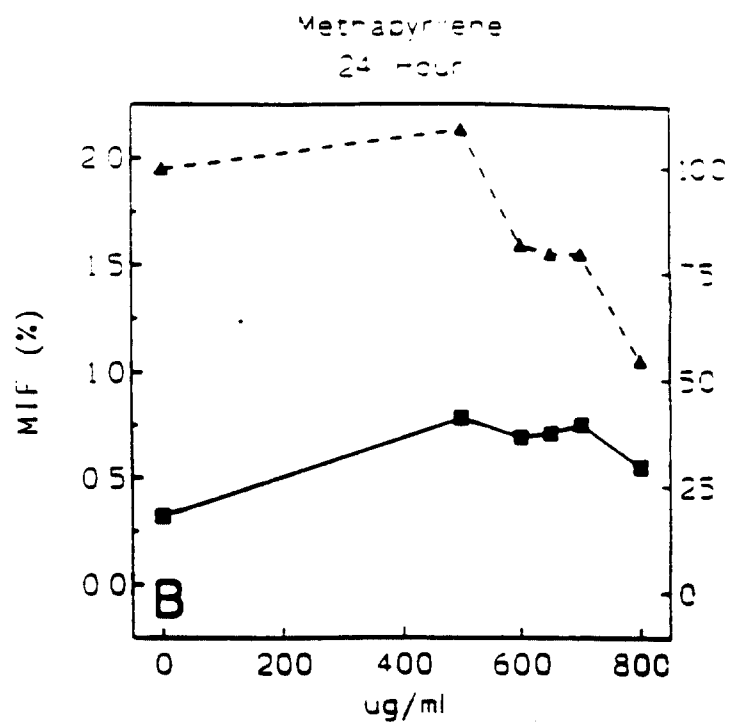
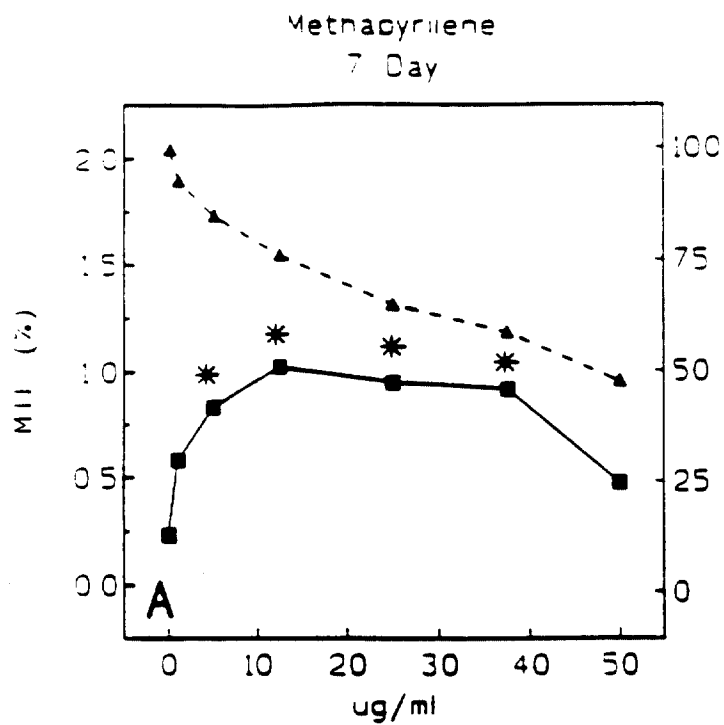


Figure 6





## Triage of 8(e) Submissions

Date sent to triage: \_\_\_\_\_

**NON-CAP**

**CAP**

Submission number: 13644 A

TSCA Inventory: **(Y)** N D

Study type (circle appropriate):

Group 1 - Gordon Cash (1 copy total)

ECO      AQUATO

Group 2 - Ernie Falke (1 copy total)

ATOX      SBTOX      SEN      w/NEUR

Group 3 -HERD (1 copy each)

STOX      CTOX      EPI      ~~RTOX~~      GTOX  
STOX/ONCO      CTOX/ONCO      IMMUNO      ~~CYTO~~      NEUR

Other (FATE, EXPO, MET, etc.): \_\_\_\_\_

Notes:

- ☒ This is the **original** 8(e) submission; refile after triage evaluation.
- ☐ This **original** submission has been **split**; rejoin after triage evaluation.
- ☐ Other:

### Photocopies Needed for Triage Evaluation

entire document: **(0)**      1      2      3

front section and CECATS:      0      1      2      3

Initials: \_\_\_\_\_

Date: \_\_\_\_\_

CBCAT/STRIAGE TRACKING DBASE ENTRY FORM

CBCAT'S DATA: Submission # 8EHQ-0596-13644 SEQ. A

TYPE (INT) SUPP FLWP

SUBMITTER NAME: Procter & Gamble

Company

INFORMATION REQUESTED: FLWP DATE: 05/01/96

0501 NO INFO REQUESTED

0502 INFO REQUESTED (TECH)

0503 INFO REQUESTED (VOL ACTIONS)

0504 INFO REQUESTED (REPORTING RATIONALE)

DISPOSITION:

0635 REFER TO CHEMICAL SCREENING

0678 CAP NOTICE

VOLUNTARY ACTIONS:

0601 NO ACTION REPORTED

0602 STUDIES PLANNED IN FUTURE

0603 NOTIFICATION OF WORK IN PROGRESS

0604 LABEL/MSDS CHANGES

0605 PROCESS/ANALYSIS CHANGES

0606 APPAUSE DISCONTINUED

0607 PRODUCTION DISCONTINUED

0608 CONFIDENTIAL

SUB. DATE: 04/30/96 OTS DATE: 05/01/96 CSRAD DATE: 06/04/96

CHEMICAL NAME: CAS# 99-55-8

<u>INFORMATION TYPE:</u>		<u>P F C</u>	<u>INFORMATION TYPE:</u>	<u>P F C</u>	<u>INFORMATION TYPE:</u>	<u>P F C</u>	
0201	ONCO (HUMAN)	01 02 04	0216	EPICLIN	01 02 04	0241	IMMUNO (ANIMAL)
0202	ONCO (ANIMAL)	01 02 04	0217	HUMAN EXPOS (PROD CONTAM)	01 02 04	0242	IMMUNO (HUMAN)
0203	CELL TRANS (IN VITRO)	01 02 04	0218	HUMAN EXPOS (ACCIDENTAL)	01 02 04	0243	CHEM/PHYS PROP
0204	MUTA (IN VITRO)	01 02 04	0219	HUMAN EXPOS (MONITORING)	01 02 04	0244	CLASTO (IN VITRO)
0205	MUTA (IN VIVO)	01 02 04	0220	ECO/AQUA TOX	01 02 04	0245	CLASTO (ANIMAL)
0206	REPRO/TERATO (HUMAN)	01 02 04	0221	ENV. OCCUR/REL/FATE	01 02 04	0246	CLASTO (HUMAN)
0207	REPRO/TERATO (ANIMAL)	01 02 04	0222	EMER INCI OF ENV CONTAM	01 02 04	0247	DNA DAM/REPAIR
0208	NEURO (HUMAN)	01 02 04	0223	RESPONSE REQUEST DELAY	01 02 04	0248	PROD/USE/PROC
0209	NEURO (ANIMAL)	01 02 04	0224	PROD/COMP/CHEM ID	01 02 04	0251	MSDS
0210	ACUTE TOX. (HUMAN)	01 02 04	0225	REPORTING RATIONALE	01 02 04	0299	OTHER
0211	CHR. TOX. (HUMAN)	01 02 04	0226	CONFIDENTIAL	01 02 04		
0212	ACUTE TOX. (ANIMAL)	01 02 04	0227	ALLERG (HUMAN)	01 02 04		
0213	SUB ACUTE TOX (ANIMAL)	01 02 04	0228	ALLERG (ANIMAL)	01 02 04		
0214	SUB CHRONIC TOX (ANIMAL)	01 02 04	0239	METAB/PHARMACO (ANIMAL)	01 02 04		
0215	CHRONIC TOX (ANIMAL)	01 02 04	0240	METAB/PHARMACO (HUMAN)	01 02 04		

TRIAGE DATA: NON-CBI INVENTORY

CAS SR YES

NO

IN HUMAN

ONGOING REVIEW

YES (DROP/REFER)

NO (CONTINUE)

REFER

SPECIES

IN VITRO

LOW

MED

HIGH

USE:

PRODUCTION:

10/1/96

14) 8EHQ-0596-13644: Rank - medium.

Chemical: 2-amino-4-nitrotoluene (CAS# 99-55-8).

A Comprehensive Protocol for Conducting the Syrian Hamster Embryo cell Transformation Assay at pH 6.70, Proctor & Gamble Co., Cincinnati OH, not dated, cover letter dated April 30, 1996: Induces morphological cell transformation, with a dose response, in Syrian hamster embryo (SHE) cells in culture.